SOY LEGHEMOGLOBIN PREPARATION: A 28-DAY DIETARY STUDY IN RATS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

DATA REQUIREMENT

OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 407): Health Effects, Repeated Dose 28-day Oral Toxicity Study in Rodents (2008)

US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. (2007)

STUDY NUMBER

43166

PERFORMING LABORATORY

Product Safety Labs
2394 US Highway 130
Dayton, New Jersey 08810

STUDY COMPLETION DATE

July 26, 2017

STUDY DIRECTOR

Mithila Shitut, BVSc & AH, MS

SPONSOR

Impossible Foods Inc.
525 Chesapeake Dr.
Redwood City, CA 94063

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Soy Leghemoglobin Preparation

This study meets the requirements of US FDA GLP: 21 CFR Part 58, 1987 and OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM (98)17, OECD, Paris, 1998. Specific information related to the characterization of the test substance(s) as received and tested is the responsibility of the study sponsor (Section 3.B) with the following exception:

1) Chemistry analysis was not conducted in compliance with GLP regulations

Specific information related to the characterization of the test substance as received and tested is the responsibility of the study Sponsor (Section 3.A).

Study Director: (b) (6) Date: 7/26/17

Name of Signer: Mithila Shitut, BVSc & AH, MS

Name of Company: Product Safety Labs

Sponsor: (b) (6) Date: 7/26/17

Name of Signer: Rachel Fraser, PhD

Name of Company: Impossible Foods Inc.

Submitter (b) (6) Date: 7/26/17

Name of Signer: Rachel Fraser, PhD

Name of Company: Impossible Foods Inc.

QUALITY ASSURANCE STATEMENT

The Product Safety Labs' Quality Assurance (QA) Unit has reviewed this final study report to assure the report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study.

QA Activities for This Study:

QA Activity	Performed By	Date Conducted	Date Findings Reported To Study Director And Management
Protocol review	R. Krick; M. Zakrzewski	Sep 14, 2016; Nov 7 & 8, 2016	Sep 14, 2016; Nov 8, 2016
In-process inspection: *Study Schedule*	M. Zakrzewski	Sep 27, 2016	Sep 27, 2016
In-process inspection: *Diet Preparation and Sampling*	M. Zakrzewski	Sep 28, 2016	Sep 28, 2016
In-process inspection: *In-life and detailed observations*	M. Zakrzewski	Oct 19, 2016	Oct 19, 2016
In-process inspection: *Necropsy*	M. Zakrzewski	Oct 27, 2016	Oct 27, 2016
Raw data audit	M. Zakrzewski	Nov 7 & 8, 2016	Nov 8, 2016
Draft report review	M. Zakrzewski	Dec 16, 2016	Dec 16, 2016

QA Statements for the chemical analysis, clinical pathology and histopathology phases of the study may be found in Appendices D, N, and T, respectively.

Final report reviewed by:

(b) (6)

Maryann Zakrzewski
Quality Assurance Auditor
Product Safety Labs

July 24, 2017
Date

CERTIFICATION

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected during the study.

(b) (6)

Mithila Shitut, BVSc & AH, MS
Study Director
Product Safety Labs

06 July 2017
Date

(b) (6)

Odete Mendes, DVM, PhD, DACVP, DABT
Director of Toxicology and Pathology
Product Safety Labs

26 July 2017
Date

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STUDY INFORMATION

Protocol No.:	P703.01 IMP
Test Substance(s):	Soy Leghemoglobin Preparation Lot #: PP-PGM2-16-088-301
Physical Descriptions:	Red/brown powder
Date Test Substance Received:	July 20, 2016
PSL IDs:	160720-5R
PSL Study Number:	43166
Sponsor:	Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063
Study Initiated-Completed:	September 21, 2016 – (see report cover page)
In-Life Study Initiated-Completed:	September 28 – October 28, 2016
Notebook No.:	16-43166: pages 1-466

KEY PERSONNEL

Product Safety Labs:

President:	Daniel J. Merkel, BS, MBA
Director of Toxicology and Pathology:	Odete Mendes, DVM, PhD, DACVP, DABT
Study Director:	Mithila Shitut, BVSc & AH, MS
Primary Scientist:	Colleen Wojenski, BS, LATG
Contributing Personnel:	Joshua Battaglia, BS Aubrey Blue Cynthia Bodnar Lisa Broske-Godin, BS, RLATG Stephanie DeCarlo, BS Janet Dell John, BA, RLATG Kathleen Quinn, BS Matthew Notta, BS Mark Schooley Matthew Sorber, BS Shannon Stevens, BS, CVT
Director of Quality Assurance:	Rhonda S. Krick, BS
Technical Writing Supervisor:	Celeste Dunn, AS

The following individual was responsible for the ophthalmology evaluations:

Ophthalmologist:	Kristina R. Vygantas, DVM, DACVO 319 Perrineville Road Robbinsville, NJ 08691

The following facility was responsible for the conduct and reporting of analysis of the neat test substance and all dietary preparations:

Test Substance and Dietary Analysis:	Impossible Foods Inc 525 Chesapeake Dr. Redwood City, CA 94063
Principal Investigator:	Rachel Fraser, PhD

KEY PERSONNEL (cont.)

The following were responsible for the clinical pathology analysis:

Clinical chemistry, hematology, coagulation and urinalysis:	DuPont Haskell Global Centers for Health and Environmental Sciences P.O. Box 30, Elkton Road Newark, Delaware 19714
Principal Investigator:	Denise Hoban, BA, MLT, ASCP
Clinical pathology evaluations:	Product Safety Labs 2394 US Highway 130 Dayton, New Jersey 08810
Principal Investigator:	Odete Mendes, DVM, PhD, DACVP, DABT

The following were responsible for the histological slide preparation and pathology evaluations:

Histological slides preparation:	HSRL Histo-Scientific Research Laboratories 5930 Main Street Mount Jackson, VA 22842
Histology Principal Investigator:	Craig Zook
Histological slide evaluation by:	HSRL Histo-Scientific Research Laboratories 5930 Main Street Mount Jackson, VA 22842
Pathology Principal Investigator:	Daniel G. Branstetter, DVM, PhD, DACVP
Histopathology Peer Review	Regan Path/Tox Services, Inc, 1457 Township Road 853 Ashland, OH 44805 P.I. (pathology): Karen Regan, DVM, DABT, DACVP

1. OBJECTIVE

The objective of this study was to evaluate the potential subchronic toxicity of Soy Leghemoglobin Preparation in male and female rats likely to arise from continuous exposure to the test substance in the diet for at least 28 days. A no-observed-adverse-effect-level (NOAEL) was also sought for each sex.

2. SUMMARY

A 28-day dietary toxicity study was conducted in Crl:SD CD® IGS rats to determine the potential of Soy Leghemoglobin Preparation to produce toxicity. Eighty (80) healthy rats (40 males and 40 females) were selected for the test and equally distributed into four groups (10/sex/group). Dietary test substance levels 512, 1024 and 1536 mg/kg/day corresponded to 250 mg/kg/day (Group 2), and 500 mg/kg/day (Group 3), and 750 mg/kg/day (Group 4) of active ingredient, as well as a Basal diet control (Group 1), were evaluated.

The neat test substance was measured to be stable under the conditions of storage over the course of this study. Stability of test substance in the diet was evaluated by analyzing the low, medium, and high dietary concentrations of the test substance on Days 0, 4, 7, and 10 following preparation. Test substance homogeneity in the diet was assessed at the beginning of the study by evaluating the low, medium, and high dietary levels in the top, middle, and bottom strata of the diet preparations. At the beginning, middle, and end of the study, selected diet preparation samples were analyzed to verify test substance concentration in the diets over the course of the study. Results from the homogeneity, stability, and concentration analyses of the test diets indicate that Soy Leghemoglobin Preparation was homogeneously distributed within an acceptable margin of variability, stable in the dietary matrix, and was considered to have met target concentrations in the diet for all intake levels.

The animals were examined by focal illumination and indirect ophthalmoscopy prior to initiation and again at the end of the study (Day 23), observed for viability, signs of gross toxicity and behavioral changes at least once daily during the study and weekly for a battery of detailed clinical observations. Body weight and food consumption measurements were collected throughout the study and used to calculate the mean overall daily intake of test substance. Urine and blood samples were collected on Day 22 from all study animals for urinalysis, hematology and clinical chemistry determinations. Gross necropsies and histological evaluation of selected organs and tissues were performed on all study animals.

Administered doses of 512, 1024 and 1536 mg/kg/day of test substance correspond to 250, 500 and 750 mg/kg/day of the active, respectively. The mean overall (Days 0-28) daily intake of the test substance in male rats fed dietary concentrations of 512, 1024 and 1536 mg/kg/day was 478.9, 954.7 and 1438.2 mg/kg/day respectivelly. For the same dietary concentrations, the mean overall (Days 0-28) daily intake in female rats was 497.8, 983.4, and 1470.4 mg/kg/day of test substance, respectively. The animals are considered to have received close to the targeted dose levels.

There were no mortalities, clinical observations, ophthalmology, body weight, body weight gain, food consumption, or food efficiency changes attributable to Soy Leghemoglobin Preparation administration.

There were no test substance related changes in hematology, serum chemistry or urinalysis parameters for males or females rats. Changes in coagulation paramentes were limited to a non dose-dependent increase in activated partial tromboplastin time observed in Group 3 and 4 males,

that due to its very slight magnitude and lack of correlating pathological or clinical finding this change is considered non adverse.

There were no microscopic or macroscopic findings related to the administration of the test substance, Soy Leghemoglobin Preparation, in male or female rats. There were no test substance-related changes in absolute or relative organ weight values in male rats treated with Soy Leghemoglobin Preparation. Decreases in uterine weight were observed in Group 2-4 female rats. These decreases did not correlate with adverse histopathological findings and are therefore interpreted to be non-adverse.

Under the conditions of the study and based on the toxicological endpoints evaluated, the no-adverse-effect level (NOAEL) for administration of Soy Leghemoglobin Preparation in the diet was determined to be 1536 mg/kg/day, which corresponds to 750 mg/kg/day of the active ingredient Soy Leghemoglobin for Sprague Dawley rats.

3. TEST SUBSTANCE

A. Source

The test substance was provided by the Sponsor.

B. Identification

The test substance was received on July 20, 2016, and identified using the following information provided by the Sponsor and Product Safety Labs (PSL) identification number.

Test Substance: Soy Leghemoglobin Preparation
PSL ID: 160720-5R
Lot #: PP-PGM2-16-088-301
Physical Description: Red/brown powder
Composition: Soy Leghemoglobin 48.82%
Storage Conditions: Frozen
Expiration Date: Not Applicable

Documentation of the methods of synthesis, fabrication, or derivation of the test substance is retained by the Sponsor.

C. Analysis

The test substance, as received, was expected to be stable for the duration of the study. Stability of the neat test substance in the dietary matrix and that of the concentration of the test substance in the test diets was determined as part of this study.

D. Hazards

Appropriate routine safety precautions were exercised in the handling of the test and control substances.

4. GENERAL TEST SYSTEM PARAMETERS

A. Animal Requirements

4.A.1 Number of Animals: 80

4.A.2 Number of Groups: 4 (3 dietary levels per sex + 1 control group per sex)

4.A.3 Number of Animals per Group: 20 (10 male, 10 female)

4.A.4 Sex: Male and female; females will be nulliparous and non-pregnant.

4.A.5 Species/Strain: CRL Sprague-Dawley CD® IGS rats

4.A.6 Age/Weight: Seven to eight weeks at initiation; the weight variation did not exceed ± 20% of the mean weight for each sex.

4.A.7 Supplier: Charles River Laboratories, Inc. Rats were shipped in filtered cartons by airfreight and/or truck.

On September 22, 2016, 88 CRL Sprague-Dawley CD® IGS rats (44 males and 44 females) arrived from Charles River Laboratories, Inc., with an assigned birth date of August 6, 2016. The rats were designated by the supplier to be 6-7 weeks of age upon arrival.

B. Test System Justification

The Sprague-Dawley® rat was the system of choice because, historically, it has been a preferred and commonly used species for dietary toxicity tests. The current state of scientific knowledge does not provide acceptable alternatives to the use of live animals to accomplish the objective of this study.

C. Animal Husbandry

4.C.1 Housing

The animals were group housed in suspended stainless steel caging, which conforms to the size recommendations in the most recent *Guide for the Care and Use of Laboratory Animals* (Natl. Res. Council, 2011). Litter paper was placed beneath the cages and was changed at least three times per week. The animal room had a 12-hour light/dark cycle and was kept clean and vermin free.

4.C.2 Animal Room Temperature and Relative Humidity Ranges

The animal room was temperature and humidity were 19-23°C and 39-62%, respectively.

4.C.3 Acclimation

The animals were conditioned to the housing facilities for six days prior to testing. Body weights and clinical observations were recorded at least two times prior to study start.

4.C.4 Feed

2016 certified Envigo Teklad Global Rodent Diet® was stored in a dedicated temperature and humidity monitored feed storage site and was available *ad libitum* during acclimation. Test diets were prepared as described in Section 6.B using 2016 certified Envigo Teklad Global Rodent Diet® and were available *ad libitum* during the study.

4.C.5 Water

Filtered tap water was available *ad libitum*. Water analysis was conducted by Precision Analytical Services, Inc., Toms River, NJ and South Brunswick Municipal Water Supply, South Brunswick, NJ.

4.C.6 Contaminants

There were no known contaminants reasonably expected to be found in the food or water that would interfere with the results of this study. Routine analysis consisting of each lot of feed used in this study was received from Envigo Teklad, Madison, WI. Water analysis was conducted periodically and the records are kept on file at Product Safety Labs. The date(s) of the most recent analyses are reported in Appendix B.

4.C.7 Viral Screen

The animals used in this study were considered to be pathogen-free as received from the vendor (Section 4.A.). Rodent-health surveillance for study animals was monitored by designating three rats as "sentinels" for the study room (Animals 257M 10.28.16, 268M 10.28.16, and 316F 10.28.16). Sentinels were housed under the conditions of the study, on racks alongside study animals, for the duration of the study (September 28 – October 28, 2016). These animals were not a part of the study, and were clearly marked as such. A serum sample was collected from each sentinel rat for screening of common rat pathogens (Rat Parvovirus, Toolan's H-1 Virus, Kilham Rat Virus, Rat Minute Virus, Parvovirus NS-1, Rat Coronavirus, Rat Theilovirus, and Pneumocystis carinii). The serum samples were sent on ice to IDEXX BioResearch (Columbia, MO) for evaluation. Serological pathogen screening results for the sentinels 257M 10.28.16, 268M 10.28.16, and 316F 10.28.16, corresponding with this study, are reported in Appendix B. The sentinel samples were negative for all pathogens evaluated and therefore, the study animals were considered to be healthy and reasonably free of common rat pathogens.

D. Identification

4.D.1 Cage

Each cage was identified by a cage card indicating the study number, dose level, group assignment, individual animal identification and sex of the animal.

4.D.2 Animal

Each animal was given a sequential number in addition to being uniquely identified with a Monel® self-piercing stainless steel ear tag.

5. EXPERIMENTAL DESIGN

A. Route of Administration

The test substance was administered in the diet.

B. Justification of Route of Administration

The dietary route of administration was used because it was recommended in the referenced guidelines (Section 8.C.), as human exposure may occur via this route.

C. Control of Bias

Animals were randomly assigned to test groups, stratified by body weight.

D. Dose Levels

Ten male and ten female test animals were randomly assigned to each of the following test groups:

Group	No. Animals/ Group M/F	Dietary Dose Level/ Target Exposure of Active Ingredient (mg/kg/day)	Dietary Dose Level/ Target Exposure of Test Substance [a] (mg/kg/day)
1	10/10	0	0
2	10/10	250	512
3	10/10	500	1024
4	10/10	750	1536

[a] Based on 48.82% active ingredient (AI, Soy Leghemoglobin) of Soy Leghemoglobin Preparationlot # PP-PGM2-16-088-301

E. Justification of Dose Level Selection

The Sponsor, in consultation with the Study Director, and based on a 14-day palatability/toxicity study (PSL, 2016) selected target dietary dose levels of 512, 1024 and 1536 mg/kg/day that correspond to target dose levels of 250, 500 and 750 mg/kg/day of the active ingredient, Soy leghemoglobin. To maintain target dietary dose levels throughout the study, concentrations in the test diets were calculated based on the most recent group body weight and food consumption data. Alternatively, historical control values, relevant to the age and weight of the rats at corresponding intervals were used. Diets for males and females at each dietary dose level were made separately each week. A NOAEL was expected to be achieved for this study.

6. GENERAL PROCEDURES

A. Selection of Animals

After acclimating to the laboratory environment for 6 days, the rats were examined for general health and weighed. Only those rats free of clinical signs of disease or injury and having a body weight range within ±20% of the mean were selected for test. Eighty (80) healthy rats (40 males; 40 females) were selected for test. The animals weighed 227-250 grams (males) and 156-198 grams (females) and were approximately 7-8 weeks of age at initiation of dosing. The rats that were used on test were randomly distributed, stratified by body weight, among the dose and control groups on the day of study start.

B. Diet Preparation and Sampling

6.B.1 Diet Preparation

The test substance was processed as needed to decrease particle size using a grinder and then added to 2016 Envigo Teklad Global Rodent Diet® and thoroughly mixed in a high-speed mixer. Control diet was mixed under the same conditions as the diets prepared with the test substance. All diets were kept frozen following preparation, unless presented to the test animals on the same day as diet preparation. All diets were prepared approximately weekly.

6.B.2 Diet Presentation

The control and test diets were presented to their respective groups on Day 0 of the study. The diets were replaced concurrently with food consumption measurements on Days 3, 7, 10, 14, 17, 21 and 24. Additional diet may be provided as needed throughout the study to insure *ad libitum* feeding. Animals were exposed to the test diets for at least 28 days.

6.B.3 Sampling

The neat test substance and selected prepared diets (at each concentration) were sampled in duplicate.

6.B.4 Stability of Test Substance

At the initial, middle, and final diet preparation, a sample of the test substance (neat) was retained for stability. Analytical results of the initial and final stability samples were used to establish the stability of the test substance under normal laboratory conditions for the duration of the study.

6.B.5 Stability in Dietary Matrix

During the first week of the study, samples to verify the stability of the test and control substance in the dietary matrix were prepared. Samples were prepared in standard feed jars with followers and retaining rings and were stored at ambient temperature in the animal room. Samples from each dietary concentration were collected at the first presentation of the diet and after 4, 7, and 10 days and frozen until analyzed.

6.B.6 Homogeneity

Samples to evaluate homogeneity of the test and control substance distribution were collected from the initial diet preparation. Samples were taken from approximately the top, middle and bottom of the diet mixer. Basal diet control samples were collected from the middle of the mixer only. Chemical analysis verified the diets as homogeneous and of accurate concentration throughout the study.

6.B.7 Concentration Verification

Samples were collected from representative animal diets of the initial (as part of the homogeneity assessment), middle and final diet preparations during which time samples were retained and stored frozen. Samples were analyzed to verify the concentration of the test diets.

6.B.8 Sample Preservation

Upon sampling, diet preparations and neat test substance were stored frozen. Samples were considered stable from the point at which they were frozen.

6.B.9 Sample Analysis

A single duplicate of the frozen diet samples described above was sent to Impossible Foods for analysis of diet preparation and neat test substance samples. A signed, analytical report was provided to the Study Director. This report included the methodology, pertinent measurements, study results, and tabulated results. Upon completion of the report, all raw data was transferred to the Study Director to be

incorporated into the main study report. Any remaining sample material was retained at Product Safety Labs until issuance of the final report.

C. Ophthalmologic Evaluations

During the acclimation period, the eyes of all rats being considered for study were examined by focal illumination, indirect ophthalmoscopy and, when indicated, slit-lamp microscopy. Mydriatic eye drops were administered prior to ophthalmoscopy and the eyes were examined in subdued light. Subdued light was maintained in the animal room. These procedures were repeated on all test animals prior to test termination on Day 23.

D. Clinical Observations

All animals were observed at least twice daily for viability. Cage-side observations of all animals were performed daily during the study. All findings were recorded.

On Day 0, prior to the first treatment with the test substance, and weekly thereafter, a detailed observation was conducted while handling the animal, generally on days that the animals were weighed and food consumption measurements were taken. Potential signs noted included, but were not limited to: changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling), or bizarre behavior (e.g., self-mutilation, walking backwards) were also recorded. The date and clock time of all observations and/or mortality checks were recorded.

E. Body Weight and Body Weight Gain

Individual body weights were recorded twice during acclimation. Test animals were weighed on Day 0 (prior to study start) and weekly thereafter (intervals of 7 days ± 1). The animals were also weighed prior to sacrifice in order to calculate organ-to-body weight ratios (Amendment 1). Body weight gain was calculated for selected intervals and for the study overall.

F. Food Consumption, Food Efficiency, and Dietary Intake of Soy Leghemoglobin Preparation

Individual food consumption was measured and recorded on Days 3, 7, 10, 14, 17, 21, 24 and at the end of the study. Food efficiency and dietary intake of the test substance (mg/kg/day) was also calculated and reported.

G. Clinical Pathology

Clinical pathology was performed on all animals for blood chemistry and hematology of the terminal sacrifice animals at the end of the dosing phase of the study. The animals were fasted overnight prior to blood collection. Blood samples for hematology (except coagulation samples) and clinical chemistry were collected via sublingual bleeding under isoflurane anesthesia during Week 4 of the test period. Approximately 500 µL of blood was collected in a pre-calibrated tube containing K_2EDTA for hematology assessments. The whole blood samples were stored under refrigeration and shipped on cold packs. Approximately 1000 µL of blood was collected into a tube containing no preservative for clinical chemistry assessments. These samples were centrifuged in a refrigerated centrifuge and the serum was transferred to a labeled tube. Serum samples were stored in a -80°C freezer and shipped frozen on dry ice. All samples were shipped to DuPont Haskell Global Centers for Health and Environmental Sciences.

The day before collection of samples for the clinical pathology evaluation, the animals were placed in metabolism cages. Animals were fasted after 3 pm (at least 15 hours prior to) and urine was collected from each animal. Urine samples were stored under refrigeration and shipped on wet ice to DuPont Haskell Global Centers for Health and Environmental Sciences.

Blood samples used to determine the prothrombin time and activated partial thromboplastin time (coagulation) were collected via the inferior vena cava under isoflurane anesthesia at terminal sacrifice. Approximately 1.8 mL of blood was collected in a pre-calibrated tube containing 3.2% sodium citrate. These samples were centrifuged in a refrigerated centrifuge and the plasma was transferred to labeled tubes. Plasma samples were stored in a -80° C freezer and shipped frozen in dry ice to DuPont Haskell Global Centers for Health and Environmental Sciences. In addition, a second blood sample was retained during the exsanguination procedure for future possible evaluation.

All blood samples were evaluated for quality by visual examination.

6.G.1 Hematology included:

erythrocyte count (RBC)
hemoglobin concentration (HGB)
hematocrit (HCT)
mean corpuscular volume (MCV)
mean corpuscular hemoglobin (MCH)
red cell distribution width (RDW)
absolute reticulocyte count (ARET)
platelet count (PLT)
total white blood cell (WBC) and differential leukocyte count
Mean corpuscular hemoglobin concentration (MCHC) was calculated.

In addition, separate, blood smears, stained with New Methylene Blue or Wright-Giemsa stain, were prepared from each animal undergoing hematological evaluation, but were not needed for examination.

6.G.2 Coagulation included:

prothrombin time (PT)
activated partial thromboplastin time (APTT)

6.G.3 Clinical chemistry included:

serum aspartate aminotransferase (AST)
serum alanine aminotransferase (ALT)
sorbitol dehydrogenase (SDH)
alkaline phosphatase (ALKP)
total bilirubin (BILI)
urea nitrogen (BUN)
blood creatinine (CREA)
total cholesterol (CHOL)
triglycerides (TRIG)
fasting glucose (GLUC)
total serum protein (TP)
albumin (ALB)
globulin (GLOB)
calcium (CALC)
inorganic phosphorus (IPHS)
sodium (NA)
potassium (K)
chloride (CL)

6.G.4 Urinalysis included:

quality (QUAL)
pH
ketone (KET)
color (COL)
glucose (UGLC)
bilirubin (UBIL)
clarity (CLAR)
specific gravity (SG)
blood (BLD)
volume (UVOL)
protein (UMTP)
urobilinogen (URO)
microscopic urine sediment examination

Any remaining serum samples were maintained frozen at approximately -80°C and discarded upon approval of the Sponsor at finalization.

H. Terminal Sacrifice and Histopathology

6.H.1 Scheduled Sacrifice

At terminal sacrifice, all survivors were euthanized by exsanguination from the abdominal aorta under isoflurane anesthesia. All animals in the study were subjected to a gross necropsy, which included examination of the external surface of the body, all orifices, musculoskeletal system, and the cranial, thoracic, abdominal, and pelvic cavities, with their associated organs and tissues. All gross lesions were recorded. The following tissues were weighed wet as soon as possible after dissection to avoid drying:

adrenals (combined)	kidneys (combined)	testes (combined)
brain	liver	thymus
epididymides (combined)	ovaries with oviducts (combined)	uterus
heart	spleen	

The following organs and tissues from all animals were preserved in 10% neutral buffered formalin for possible future histopathological examination:

accessory genital organs (prostate and seminal vesicles)	ileum with Peyer's patches	rectum
	jejunum	salivary glands (sublingual
adrenals	kidneys	submandibular, and parotid)
all gross lesions	larynx	skeletal muscle
aorta	liver	skin
bone (femur)	lungs	spinal cord – 3 levels:
bone marrow (from femur & sternum)	lymph node mandibular	cervical, mid-thoracic, and lumbar
	lymph node mesenteric	
brain – 3 sections including medulla/pons, cerebellar, and cerebral cortex	mammary gland	spleen
	nasal turbinates	sternum
	nose	stomach
cecum	ovaries	thymus
cervix	oviducts	thyroid
colon	pancreas	trachea
duodenum	parathyroid	urinary bladder
esophagus	peripheral nerve (sciatic)	uterus
Harderian gland	pharynx	vagina
heart	pituitary gland	

The following organs and tissues from all animals were preserved in modified Davidson's fixative and then stored in ethanol for possible future histopathological examination:

eyes	optic nerve
epididymides	testes

Additional tissues were preserved if indicated by signs of toxicity or target organ involvement.

6.H.2 Histopathology

Histological examination was performed on the preserved organs and tissues of the animals from both the control and high dose groups (Groups 1 and 4, respectively). The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin (HE) and examined by light microscopy. Additional special stains can be added based on HE evaluation at the discretion of the study pathologist in consultation with the study director and sponsor. Slide preparation and histological assessment, by a board-certified veterinary pathologist, was performed at Histo-Scientific Research Laboratories (HSRL).

6.H.3 Histopathology Peer Review

A histopathology peer review of female reproductive organs was performed for all female rats (Ammendment 3). The peer review pathologist was Karen Regan, DVM, DABT, DACVP form Regan Path/Tox Services, Inc, 1457 Township Road 853, Ashland, OH 44805. A peer review statement will be inserted in Appendix U.

7. STATISTICAL ANALYSIS

Product Safety Labs performed statistical analysis of all data collected during the in-life phase of the study as well as organ weight data. DuPont Haskell Global Centers for Health and Environmental Services provided analysis of clinical pathology results to Product Safety Labs. The use of the word "significant" or "significantly" indicates a statistically significant difference between the control and the experimental groups. Significance was judged at a probability value of $p<0.05$. Male and female rats were evaluated separately.

A. Statistical Methods (In-Life and Organ Weight Data)

Mean and standard deviations were calculated for all quantitative data. If warranted by sufficient group sizes, data within groups were evaluated for homogeneity of variances and normality by Bartlett's test (Bartlett, 1937). Where Bartlett's test indicated homogeneous variances, treated and control groups were compared using a one-way analysis of variance (ANOVA). When one-way analysis of variance was significant, a comparison of the treated groups to control by Dunnett's test (Dunnett, 1964, 1980) for multiple comparisons was performed. Where variances were considered significantly different by Bartlett's test, groups were compared using a non-parametric method (Kruskal-Wallis non-parametric analysis of variance; Kruskal and Wallis, 1952). When non-parametric analysis of variance was significant, comparison of treated groups to control was performed using Dunn's test (Dunn, 1964). Statistical analysis was performed on all quantitative data for in-life and organ weight parameters using Provantis® version 9, Tables and Statistics, Instem LSS, Staffordshire UK.

B. Statistical Methods (Clinical Pathology)

Significance was judged at a probability value of $p < 0.05$. Males and females were analyzed separately (Provantis™ version 8, Tables and Statistics, Instem LSS, Staffordshire UK).

		Method of Statistical Analysis	
Parameter	Preliminary Test	If preliminary test is not significant	If preliminary test is significant
Clinical Pathology[a]	Levene's test for homogeneity and Shapiro-Wilk test for normality	One-way analysis of variance followed with Dunnett's test	Transforms of the data to achieve normality and variance homogeneity were used. The order of transforms attempted was log, square-root, and rank-order. If the log and square-root transforms failed, the rank-order was used.

[a] When an individual observation is recorded as being less than a certain value, calculations are performed on half the recorded value. For example, if bilirubin is reported as <0.1, 0.05 is used for any calculations performed with that bilirubin data. When an individual observation is recorded as being greater than a certain value, calculations are performed on the recorded value. For example, if specific gravity was reported as >1.100, 1.100 is used for any calculation performed with that specific gravity data.

8. STUDY CONDUCT

A. Laboratory

In-life portion	Product Safety Labs 2394 US Highway 130 Dayton, NJ 08810
Ophthalmology evaluation	Kristina R. Vygantas, DVM, DACVO 319 Perrineville Rd. Robbinsville, NJ 08691
Clinical chemistry, hematology, coagulation, and urinalysis	Dupont Haskell Global Centers for Health and Environmental Sciences P.O. Box 30 Elkton Road Newark, DE 19714 P.I.: Denise Hoban, BA, MLT, ASCP
Clinical pathology evaluation	Product Safety Labs 2394 US Highway 130 Dayton, NJ 08810 P.I.: Odete Mendes, DVM, PhD, DACVP, DABT
Test substance and dietary analysis	Impossible Foods Inc 525 Chesapeake Dr. Redwood City, CA 94063 P.I.: Pavel Aronov, PhD
Histological slide preparation	Histo-Scientific Research Laboratories (HSRL) 5930 Main Street Mount Jackson, VA 22842 P.I. (histology): Craig Zook

Histological slide evaluation	Histo-Scientific Research Laboratories (HSRL) 5930 Main Street Mount Jackson, VA 22842 P.I. (pathology): Daniel G. Branstetter, DVM, PhD, DACVP
Histopathology Peer Review	Regan Path/Tox Services, Inc, 1457 Township Road 853 Ashland, OH 44805 P.I. (pathology): Karen Regan, DVM, DABT, DACVP

B. GLP Compliance

This study was conducted in compliance with the following regulations:

- U.S. FDA GLP: 21 CFR Part 58, 1987

Which is compatible with:

- OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM (98)17, OECD, Paris, 1998.

Clinical pathology assessment was conducted in compliance with U.S. FDA GLP: 21 CFR Part 58, 1987 which is compatible with OECD Good Laboratory Practices.

Analysis of the neat test substance and test substance in the dietary matrix, for homogeneity, stability, and dose concentration verification, were performed in a non-GLP certified facility.

C. Test Procedure Guidelines

This study design was based on the following guidelines:

- OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 407): Health Effects, *Repeated Dose 28-day Oral Toxicity Study in Rodents* (2008).

- US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. (2007).

9. FINAL REPORT AND RECORDS TO BE MAINTAINED

The original, signed final report was sent to the Sponsor. A copy of the signed report, together with the protocol and all raw data generated at Product Safety Labs, will be maintained in the Product Safety Labs Archives. PSL will maintain these records for a period of at least five years. After this time, the Sponsor of the study will be offered the opportunity to take possession of the records or request continued archiving by PSL.

The following records are maintained:

A. Information on test substance includes but is not limited to the following:

Storage	Dietary analysis
Usage	Test substance analysis
Disposition	

B. Information on animals includes but is not limited to the following:

Receipt, date of birth	Clinical observations
Initial health assessment	Histopathology data
Dosing	Individual necropsy records
Body weights	Organ weights
Food consumption	Ophthalmologic evaluations

Hematology, clinical chemistry, coagulation, urinalysis data

All other records that would demonstrate adherence to the protocol.

Raw data related to hematology and clinical chemistry evaluations will be maintained by Product Safety Labs and/or DuPont Haskell Global Centers for Health and Environmental Sciences, Newark, DE. Prepared slides and pathology data will be maintained by Product Safety Labs and/or by HSRL, 5930 Main Street, Mount Jackson, VA, 22842. Test substance and dietary analysis data will be maintained by Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063.

10. PROTOCOL AND PROTOCOL AMENDMENTS

See Appendix A for the Protocol and Protocol Amendments.

11. RESULTS

A. Test Substance and Diet Analysis (Table 1A-D, Appendix D)

The test substance was expected to be stable under the conditions of storage over the course of this study.

11.A.1 Analysis of Soy Leghemoglobin Preparation Neat Test Substance

Soy Leghemoglobin Preparation was found to be stable under the conditions of storage over the course of this study. Results of the stability analysis of Soy Leghemoglobin Preparation from Day 0 to Day 21, found a change of -4.30%, for an overall test substance stability of 95.70% over the course of the study, within the range of analytical variance of measured test substance.

11.A.2 Stability

Dietary stability samples collected after 10 days of storage were compared to the initial samples for overall in-room stability of the test substance in the dietary matrix. All dietary mixtures were found to be stable within an acceptable degree of variation. The results of the stability were 90.74, 96.65, and 97.77% and 99.63, 93.78, and 97.38% on Day 10 of the nominal concentrations of 250, 500, and 750 mg/kg/day Soy Leghemoglobin Preparation for Groups 2-4 males and females, respectively.

11.A.3 Homogeneity

A sampling from the top, middle, and bottom of the dietary preparations found all dietary mixtures to be homogeneously distributed within an acceptable degree of variation. Analysis of the top, middle, and bottom of the dietary preparations resulted in a relative standard deviation (RSD) of 2.92, 3.09, and 5.24% and 4.77, 5.50, and 5.57% between the strata, for concentrations of 512, 1024, and 1536 mg/kg/day Soy Leghemoglobin Preparation, which corresponds to 250, 500, and 750 mg/kg/day of active ingredient for Groups 2-4 males and females, respectively.

11.A.4 Concentration Verification

Concentration verification results for Day 0 (obtained from the homogeneity analysis) averaged 92.86, 93.13, and 103.35% and 97.28, 98.53, and 100.35% for 250, 500, and 750 mg/kg/day Soy Leghemoglobin Preparation for Groups 2-4 males and females, respectively. Day 21 resulted in 93.24, 97.05, and 94.73% and 92.80, 97.76, and 97.65% for 250, 500, and 750 mg/kg/day Soy Leghemoglobin Preparation for Groups 2-4 males and females, respectively.

Based on the stability, homogeneity, and dose concentration verification results, the animals are considered to have received the targeted dietary concentrations of Soy Leghemoglobin Preparation, with an acceptable margin of variability.

B. Ophthalmologic Examinations (Appendix E)

Both eyes of all animals on study were examined by focal illumination, slit lamp biomicroscopy, and indirect ophthalmoscopy prior to study initiation and near termination of the study (Day 23). All animals included in the study were normal upon ophthalmic exam. Therefore, the test substance was not considered an ocular toxicant.

C. Mortality and Clinical Observations (Tables 2 and 3, Appendices F-H, and O)

No mortalities were observed during this study. There were no clinical observations attributable to the administration of Soy Leghemoglobin Preparation.

Males

Incidental in-life clinical observations included: red staining in the litter tray of 7/10 Group 4 animals and superficial eschar of the head in 1/10 Group 4 animals.

There were no detailed clinical observations noted for any male during the study.

Females

Incidental in-life clinical observations included: slight to moderate alopecia on the left/right forelimb in 1/10 Group 2 animals.

Incidental detailed clinical observations corresponding to the daily findings included hair loss in 1/10 Group 2 animals.

The fate of all animals is presented in Appendix O.

D. Body Weight and Body Weight Gain (Tables 4 and 5, Appendices I and J)

There were no body weight or body weight gain findings considered attributable to Soy Leghemoglobin Preparation administration.

Males

Mean body weights and mean daily bodyweight gain for the treated male rats in Groups 2-4 were comparable to the control Group 1 values throughout the study.

Females

Mean body weights for the treated female rats in Groups 2-4 were comparable to the control Group 1 values throughout the study.

Mean daily body weight gain for the treated female rats in Groups 2-4 was generally comparable to the control Group 1 values throughout the study with the exception of a transient statistically significant decrease ($p < 0.01$) in Group 2 mean daily body weight gain on Days 14-21 that was interpreted to have no toxicological relevance.

E. Food Consumption, Food Efficiency, and Dietary Intake of Soy Leghemoglobin Preparation (Tables 6-8, Appendices K-M)

There were no food consumption or food efficiency findings considered attributable to Soy Leghemoglobin Preparation administration.

Males

Mean daily food consumption for the treated male rats in Group 2-4 was generally comparable to the control Group 1 values throughout the study with the exception of significant increases ($p <$ 0.05-0.01) in Group 3 on Days 7-14 and in Group 4 on Days 7-10, that we transient and without significant inpact on body weight and are interpreted to be non-toxicologically relevant

Mean food efficiency for the treated male rats in Group 2-4 was comparable to the control Group 1 values throughout the study.

Females

Mean daily food consumption for the treated female rats in Group 2-4 was comparable to the control Group 1 values throughout the study.

Mean food efficiency for the treated female rats in Group 2-4 was generally comparable to the control Group 1 values throughout the study, with the exception of statistically significant increases ($p < 0.01$) in Group 2 on Days 14-21 that were transient and without significant inpact on body weight and are interpreted to be non-toxicologically relevant.

Dietary Intake

Administered doses of 512, 1024 and 1536 mg/kg/day of test substance correspond to 250, 500 and 750 mg/kg/day of the active, respectively. The mean overall (Days 0-28) daily intake of the test substance in male rats fed dietary concentrations of 512, 1024 and 1536 mg/kg/day was 478.9, 954.7 and 1438.2 mg/kg/day respectivelly. For the same dietary concentrations, the mean overall (Days 0-28) daily intake in female rats was 497.8, 983.4, and 1470.4 mg/kg/day of test substance, respectively. The animals are considered to have received close to the targeted dose levels.

F. Clinical Pathology (Tables 9-12, Appendix N)

11.F.1 Hematology

There were to no test substance related changes in hematology parameters for males or females rats.

Other differences in hematology values that were statistically significant are listed below. These were observed in a non-dose dependent manner and are interpreted to be within expected biological variation and are not toxicologically relevant:

- Increased Red blood cell, hemarocrit and Hemoglobin values and absolute basophil counts in Group 2 females.
- Decreased absolute reticulocyte counts in Group 3 females.

11.F.2 Coagulation

There were no test substance related changes in coagulation parameters for female rats.

A non dose dependend increase in activated partial tromboplastin time was observed in Group 3 and 4 males. Due to its very slight magnitude and lack of correlating pathological or clinical finding this change is considered non adverse.

11.F.3 Clinical Chemistry

There were no test substance related changes in serum chemistry parameters for male rats.

Decreased alkaline phosphatase was minimally decreased in a non dose dependent manner for females at all dose levels. This minimal decrease was not correlated with concurrent clinical pathology or histopathology changes and due to its limited clinical relevance is interpreted to have no toxicological significance.

Other differences in serum chemistry parameters that were statistically significant are listed below. These were observed in a non-dose dependent manner and are interpreted to be within expected biological variation and are not toxicologically relevant:

- Increased albumin and potassium values in Group 3 males.
- Decreased glucose and chloride in Groups 2 and 3 females.
- Increased globulin values in Group 3 females.
- Increased calcium in Groups 2 and 3 females.

11.F.4 Urinalysis

There were no test substance related changes in urinalysis parameters for males or female rats.

In summary, there were to no test substance related changes in hematology, serum chemistry or urinalysis parameters for males or females rats. Changes in coagulation paramenters were limited to a non dose dependent increase in activated partial tromboplastin time observed in Group 3 and 4 males, that due to its very slight magnitude and lack of correlating pathological or clinical finding this change is considered non-adverse.

G. Sacrifice, Macroscopic Observations, and Histopathology (Tables 13-16, Appendices O-T)

There were no microscopic or macroscopic findings related to the administration of the test substance, Soy Leghemoglobin Preparation, in male or female rats. There were no test substance-related changes in absolute or relative organ weight values in male rats treated with Soy Leghemoglobin Preparation. Decreases in uterine weight were observed in Group 2-4 female rats. These decreases did not correlate with adverse histopathological findings and are therefore interpreted to be non-adverse.

11.G.1 Macroscopic

There were no early deaths among the animals submitted for histopathological evaluation.

Males

Incidental necropsy observations included: a small soft right testicle and small right epididymis in 1/10 Group 1 animals.

Females

Incidental necropsy observations included: spleen stricture in 1/10 Group 3 animals and a fluid filled uterus in 4/10 Group 1 and 1/10 Group 3 animals.

At the Day 29/30 time point, there were no macroscopic findings related to the administration of the test substance, Soy Leghemoglobin Preparation, in male or female rats. In the female rats, the presence of "fluid filled" uteri (which correlated with dilation), typically associated with normal proestrus stage of the estrous cycle, was decreased in rats treated with 512 and 1536 mg/kg/day Soy Leghemoglobin Preparation. Fluid filled uteri were noted in 4 out of 10 females at 0 mg/kg/day (Group 1 Animals 7013, 7017, 7018, and 7020), in 0 out of 10 females at 512 mg/kg/day, in 1 out of 10 females at 1024 mg/kg/day (Group 3 Animal 7053), and in 0 out of 10 females at 1536 mg/kg/day. Fluid filled uteri correlated with the proestrus stage of the estrus cycle, and higher individual uterine weights, and is a normal finding with this stage of the cycle. The decreased macroscopic incidence of fluid filled uteri in treated female rats correlated with lower incidences of proestrus, resulting in significantly decreased uterine weights in the 512 and 1536 mg/kg/day groups. Notably, the incidences of animals in metestrus in the treated groups were not dose-related.

The remaining macroscopic observations at the Day 29/30 time point were also of sporadic incidence and showed no trends/patterns to suggest a relationship to administration of Soy Leghemoglobin Preparation. These findings included testis and epididymis small and/or soft right which had a microscopic correlate of atrophy and aspermia, respectively, in control group Animal 7002; brain depressed area, which was an artifact confirmed microscopically, in Group 3 Animal 7047; and spleen stricture, with no microscopic correlate, in Group 3 Animal 7055.

11.G.2 Microscopic

At the Day 30 time point, there were no Soy Leghemoglobin Preparation-related effects.

There was a decrease in the incidence of dilated uterine lumens in the 536 and 1536 mg/kg/day rats compared to controls. The uteri were dilated in 4 out of 10 females at 0 mg/kg/day (Animals 7013, 7017, 7018, and 7020), which was consistent with proestrus/estrus. There were no females with dilated uterine lumens in the 512 and 1536 mg/kg/day rats and two out of 8 in the 1024 mg/kg/day group (Animals 7053 and 7059), which correlated with lower incidences of animals in the proestrus/estrus stage of the estrus cycle. Microscopically, 512 and 1536 mg/kg/day rats tended to be in the metestrus stage of the estrous cycle, which correlated with the lower weights and was an unusual distribution. However, the presence of both new and old corpora lutea in females from all groups indicates that these females were cycling normally and there were no treatment related effects on the estrus cycle.

All other microscopic findings at the Day 29/30 time point were unrelated to administration of Soy Leghemoglobin Preparation and can be observed in the age and strain of rats used in this study.

11.G.3 Organ Weights and Ratios

There were no test substance-related changes in absolute or relative organ weight values in male rats treated with Soy Leghemoglobin Preparation. Decreases in uterine weight were observed in Group 2-4 female rats. These decreases did not correlate with adverse histopathological findings and are therefore interpreted to be non-adverse.

Males

Mean absolute and relative organ weights for Groups 2-4 were comparable to control Group 1 values throughout the study.

Females

Mean absolute and relative organ weights for Groups 2-4 were generally comparable to control Group 1 values throughout the study with the exception of decreases in mean absolute and relative uterus weights in Groups 2-4 that were statistically significant ($p <$ 0.05-0.01) in Group 2 and Group 4 animals.

12. CONCLUSION

There were no mortalities, clinical observations, ophthalmology, body weight, body weight gain, food consumption, or food efficiency changes attributable to Soy Leghemoglobin Preparation administration.

There were no test substance related changes in hematology, serum chemistry or urinalysis parameters for males or females rats. Changes in coagulation paramenters were limited to a non dose dependend increase in activated partial tromboplastin time observed in Group 3 and 4 males, that due to its very slight magnitude and lack of correlating pathological or clinical finding this change is considered non adverse.

There were no microscopic or macroscopic findings related to the administration of the test substance, Soy Leghemoglobin Preparation, in male or female rats. There were no test substance-related changes in absolute or relative organ weight values in male rats treated with Soy Leghemoglobin Preparation. Decreases in uterine weight were observed in Group 2-4 female rats. These decreases did not correlate with adverse histopathological findings and are therefore interpreted to be non-adverse.

Under the conditions of the study and based on the toxicological endpoints evaluated, the no-adverse-effect level (NOAEL) for administration of Soy Leghemoglobin Preparation in the diet was determined to be 1536 mg/kg/day, which corresponds to 750 mg/kg/day of the active ingredient Soy Leghemoglobin for Sprague Dawley rats.

13. REFERENCES

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